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Research paper

Microbial hydrogen production from depleted hydrocarbon reservoirs: Evidence from field investigations and lab experiments

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ABSTRACT

The study explores microbial Hydrogen (H_2) production in depleted hydrocarbon fields through field investigation and controlled experimental analyses (microbial process) using in-situ microbes, which thrive in thermophilic anerobic conditions of the hydrocarbon reservoirs. Gas and brine samples from various wells revealed H_2 presence in the range of 2–20 %, indicating microbial H_2 production potential in depleted hydrocarbon fields. Brine samples were characterized for their geochemical properties. Metabarcoding identified Clostridium as the most efficient H_2 producer, with other microbes including Hydrogenophaga, Desulfovibrio, Geobacter, and Thermomicrobium. In laboratory experiments, microbial cultures produced considerable hydrogen when grown in an anaerobic basal medium (ABM) enriched with 10 % v/v brine and 1 % v/v oil. The addition of oil significantly improved both the growth of the microbes and their hydrogen yield, providing valuable insights into the mechanisms of microbial hydrogen generation in hydrocarbon reservoirs.

1. Introduction

Amid growing concerns about climate change, many countries are moving away from fossil fuels toward cleaner energy sources. As natural and fossil fuel reserves dwindle and the environmental damage they cause becomes more evident, the search for sustainable alternatives has intensified. H_2, in particular, is gaining attention as a key player in this energy transition. It holds promise for decarbonizing tough-to-electrify sectors such as heavy industry, shipping, aviation, and power generation(Boshagh and Rostami, 2020; Chen et al., 2023). Furthermore, the recent global energy crisis has underscored H_2's potential not only as a low-carbon fuel but also as a means to enhance energy security prompting many governments to weave H_2 into their net-zero strategies (Alotaibi et al., 2020; Dehghanimadvar et al., 2020; Wang and Azam, 2024; Yi et al., 2023).

Major economies are advancing industrial policies to accelerate the development and deployment of H_2 technologies. However, despite growing momentum, widespread adoption remains slow due to persistent challenges. Several H_2 production methods present significant opportunities for advancement. According to Sivaramakrishnan et al. (2021), approximately 55 million tons of H_2 are produced annually, with usage increasing year after year. Recent data indicates that H_2 production reached 97 million metric tons in 2023, with less than 1 % attributed to emissions. Despite this progress, renewable H_2 production remains one and a half to six times more expensive than fossil-based methods. Additionally, around 40 % of planned low-emission H_2 projects are located in water-stressed regions, raising concerns about water resource management.

At present, nearly 95 % of H_2 is produced from natural gas using techniques like steam methane reforming, coal gasification, and water electrolysis (Nazir et al., 2020; Boretti and Banik, 2021). However, these methods are both expensive and highly energy-intensive, which raises sustainability concerns (Hallenbeck, 2011; Kotay and Das, 2010; Mokheimer et al., 2024; Qureshi et al., 2023).

In contrast, producing H_2 through microbial processes shows significant promise. Not only does it generate lower emissions, but it also has the potential to reduce production costs. As Dincer and Acar (2015) note, it's important to consider trade-offs among cost, efficiency, reliability, and scalability when choosing a H_2 production method. Evaluating these factors can guide us toward more sustainable pathways for H_2 production.

Over the past few decades, researchers have extensively investigated

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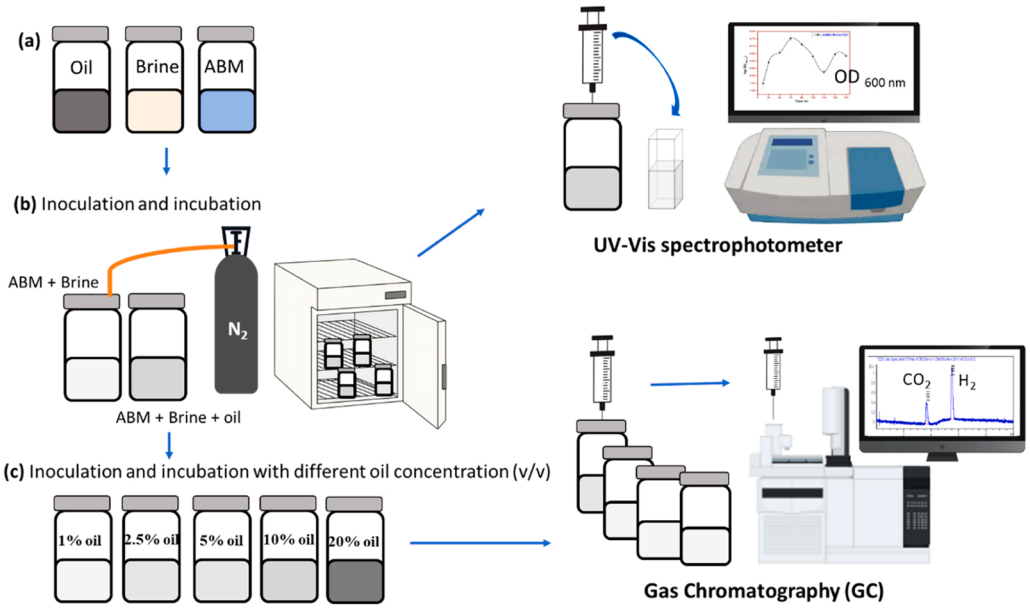

Fig. 1. Experimental approach for microbial hydrogen production in the depleted hydrocarbon field in this study.

the production of H_2 using microbial methods (Noike et al., 2002; Niu et al., 2010; Sanchez-Torres et al., 2013; Chookaew et al., 2014; Tran et al., 2014; Park et al., 2016; Pugazhendhi and Thamaraiselvi, 2017; Mirzoyan et al., 2017; Gevorgyan et al., 2018; Andreani et al., 2019; Savla et al., 2020; Sivaramakrishnan et al., 2021; Rathi et al., 2023; Goria et al., 2024; Jiao et al., 2024; Kumar et al., 2024) and most recent Vilcáez and Chowdhury (2025). A variety of fermentative microbes have been studied for their H_2-producing capabilities. For example:

- **Clostridium species** (such as *C. cellulovorans* and *C. acetobutylicum*) produce H_2 under strictly anaerobic conditions (Valdez-Vazquez et al., 2019).
- **Enterobacter species** (e.g., *Enterobacter aerogenes*) are effective under facultative anaerobic conditions, making them versatile for different environments (Lee et al., 2014; Lopes et al., 2015).
- **Escherichia coli** is popular because of its high metabolic activity and ease of genetic manipulation, which can boost H_2 yields (Sanchez-Torres et al., 2013; Tran et al., 2014; Mirzoyan et al., 2017; Gevorgyan et al., 2018).
- **Klebsiella species**, particularly *Klebsiella pneumoniae*, have also been shown to efficiently produce H_2 when culture conditions like pH and glucose concentration are optimized (Niu et al., 2010; Chookaew et al., 2014; Pugazhendhi and Thamaraiselvi, 2017).

Other notable contributors include:

- **Bacillus species** (notably *Bacillus firmus*), which are known for their robust growth even under harsh conditions (Gutiérrez-García et al., 2020).
- **Thermophilic microorganisms** such as *Thermoanaerobacterium*, *Thermosaccharolyticum*, and *Thermotoga neapolitana*, which thrive at high temperatures and tend to yield higher amounts of H_2 (Algapani et al., 2016; Bibra et al., 2018).
- **Lactic acid bacteria** (like *Lactobacillus* and *Sporolactobacillus*) can also produce H_2, though their productivity is sometimes limited by lactic acid accumulation (Andreani et al., 2019; Noike et al., 2002; Park et al., 2016).

Furthermore, thermophilic anaerobes typically yield moderate amounts of H_2 (maximum 4 mol H_2/mol of substrate) when utilizing substrates like glucose (Panagiotopoulos et al., 2015). Hydrogen yield (kilograms of H_2 per ton of dry biomass) was estimated by a two-step dark and photofermentation method, normalized to a scale of 0–100, assuming theoretical H_2 yields of 8, 4, and 3.3 moles per mole of sucrose, glucose, and xylose, respectively (De Vrije et al., 2010). In contrast, obligate anaerobes such as *Clostridium acetobutylicum*, *C. beijerinckii*, and *C. butyricum* can achieve yields up to 2.81 mol H_2 per mol of glucose through fermentative pathways.

Whereas, Thermophilic microorganisms, such as *Thermoanaerobacterium thermosaccharolyticum* and *Thermotoga neapolitana*, thrive at high temperatures (45–80°C), accelerating metabolic rates and achieving superior H_2 yields (up to 2.64 mol H_2/mol glucose), particularly from cellulose and lignocellulosic waste as substrate. Conclusively, facultative anaerobes offer flexibility but lower efficiency, obligate anaerobes provide high yields but are oxygen-sensitive, while thermophiles ensure high production and minimal contamination. Studies show that H_2 production yield is influenced by pH, organic loading rate (OLR), temperature, inoculum source, and pre-treatment (Liu and Shen, 2004; Vasconcelos et al., 2016; Marone et al., 2017; Kovalev, 2021; Laikova et al., 2023). Liu and Shen (2004) addressed several crucial parameters during batch studies, including initial pH, iron content, nitrogen concentration (NH_4HCO_3), and starch concentration when utilizing mixed bacteria, principally *Clostridium pasteurianum*. Furthermore, anaerobic H_2 production typically involves dark fermentation, where microorganisms break down organic matter (such as glucose or other carbohydrates) in the absence of oxygen to produce H_2, carbon dioxide (CO_2), and various volatile fatty acids. The general stoichiometric equation for the fermentation of glucose ($C_6H_{12}O_6$) by anaerobic bacteria (such as Clostridium species) is shown in Eqs. 1, 2, and 3 respectively (Saravanan et al., 2021).

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2 \quad (1)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2)$$

$$C_6H_{12}O_6 + 6H_2O \rightarrow 2CO_2 + CH_3CH_2CH_2COOH + 2H_2 \quad (3)$$

In addition, (Zengler et al., 1999) demonstrated that the conversion of n-$C_{16}H_{34}$ to CH_4 can be achieved by hydrocarbon-degrading and H_2-producing bacteria (HDeHPB) that decompose n-$C_{16}H_{34}$ to H_2 according to Eq. 4.

$$_4C_{16}H_{34} + 64H_2O \rightarrow 32CH_3COO^- + 32H^+ + 68H_2 \quad (4)$$

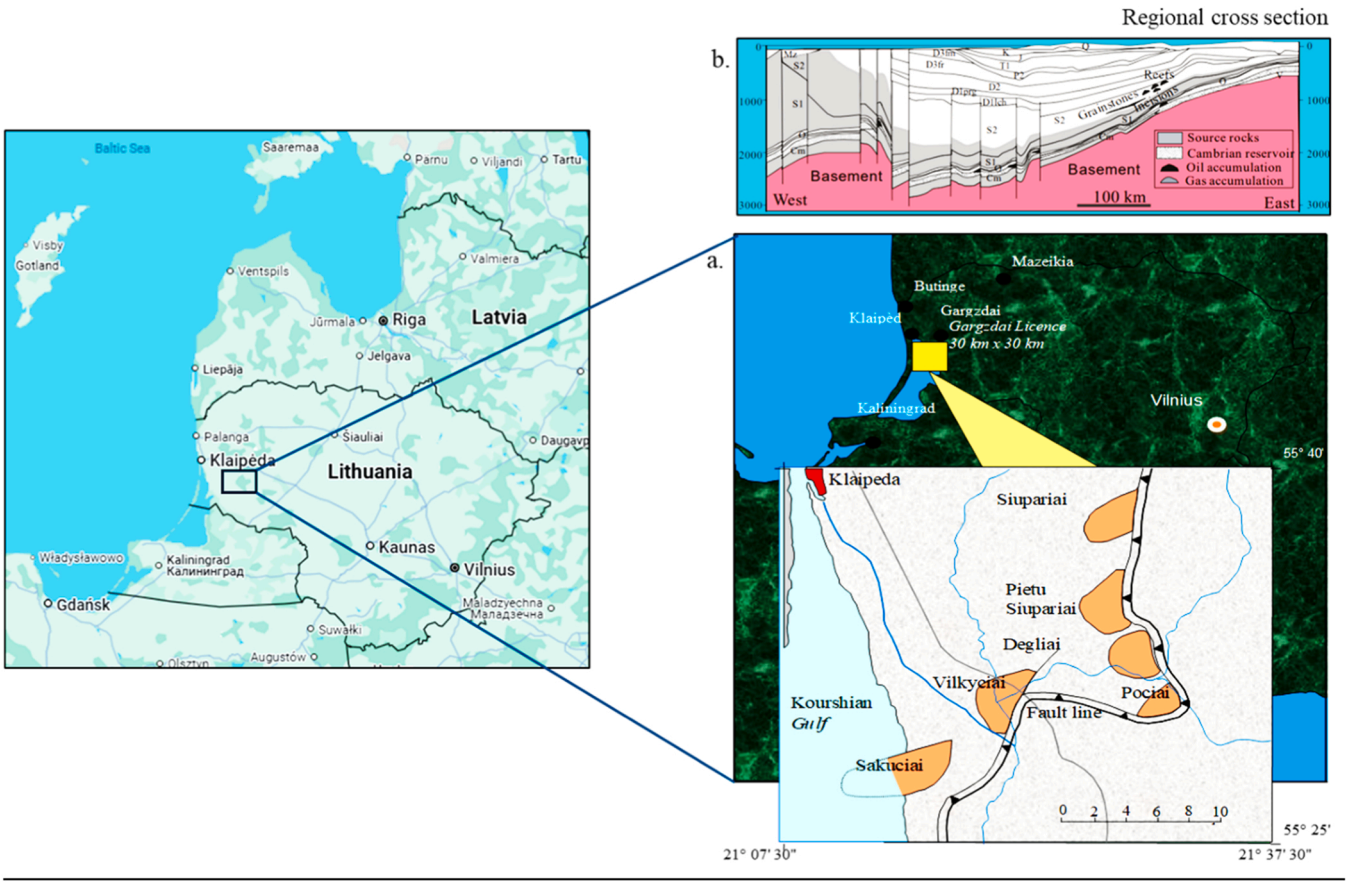

Fig. 2. Geological map depicting the sampling area and field location of a) Oil fields, Including the Pietų Šiūparių fields, and b) regional cross-section.

Moreover, the biodegradation of C_7H_8 (an aromatic compounds) to H_2 by HDeHPB via Eq. 5 was reported by Edwards and Grbic-Galic (1994)

$$C_7H_8 + 9H_2O \rightarrow HCO_3^- + 3CH_3COO^- + 4H^+ + 6H_2 \quad (5)$$

Literature reviews reveal diverse potential of microbial processes for H_2 production, which could play an important role in the development of sustainable energy systems. H_2 production from crude oil using microbial processes is an emerging concept, though it is still in the early stages of development. Other than microbial processes, H_2 production from crude oil is well explored using thermolysis (Sinitsin et al., 2023) and pyrolysis (Davidian et al., 2007) etc. Moreover, Djimasbe et al. (2022) explored H_2 production from extra-heavy crude oil using Ni-Co/Al_2O_3 catalysts under supercritical water (SCW) conditions. A decade ago, Sugai et al., (2012) explored the H_2 productivity of hydrocarbon-degrading, H_2-producing bacteria (HDeHPB) in oil reservoirs. This study shows that microbial processes can produce H_2 by metabolizing the hydrocarbons in depleted crude oil reserves. Likewise, Veshareh et al., (2022a) reported that *Thermotoga* strains convert hydrocarbons to H_2, with DSM 9442 yielding 1.02 mmol H_2/L from crude oil, while adding Tween 80 and glucose-boosted production 12-fold. Long ago, studies by Edwards and Grbic-Galic (1994) and Zengler et al., (1999) established that oil-degrading and H_2-producing thermophilic bacteria (ODHPTB) can efficiently decompose hydrocarbons into H_2.

Besides this, there is limited information available in the literature on H_2 production in depleted hydrocarbon fields. This necessitates a comprehensive investigation of microbial communities present in depleted oil fields. Additionally, the optimal conditions for the growth and H_2 production of identified microbial species in reservoir brine need to be investigated, including factors such as temperature, pH, and nutrient availability.

Therefore, through this study, an attempt has been made to address some crucial aspects of microbial H_2 production based on field and experimental investigation. The present study aims to a) Evaluate and characterize gas and brine samples extracted from depleted hydrocarbon fields and b) Investigate H_2 producing microbial species in the extract from depleted crude oil fields. c) To assess the productivity of ODHPTB in reservoir brine, and the role of Anearobic Basal Media (ABM) in enhancing H_2 production. Addressing these gaps will effectively optimize microbial H_2 production in depleted crude oil fields, and can contribute to the advancement of clean energy technologies.

2. Methodology

This section overviews the hydrocarbon fields and outlines the rationale for exploring H_2 production from depleted reservoirs. It then summarizes the laboratory experiments conducted to produce H_2 from a depleted hydrocarbon field. Additionally, the ABM (Anaerobic Basal Media) composition and the experimental methodology are described in detail. Fig. 1 illustrates a step-by-step experimental process for microbial H_2 production using oil, brine, and ABM. The process includes preparation, incubation under nitrogen conditions, oil content variation, microbial development examination using optical density (OD) estimation, and gas composition testing (detection of H_2 and CO_2) using gas chromatography.

2.1. Hydrocarbon field

Geological map showing the location of oil fields (Fig. 2). The area is structurally complex, dominated by regional fault systems such as the Klaipėda and Vilkyčiai faults, which create fault-bounded compartments

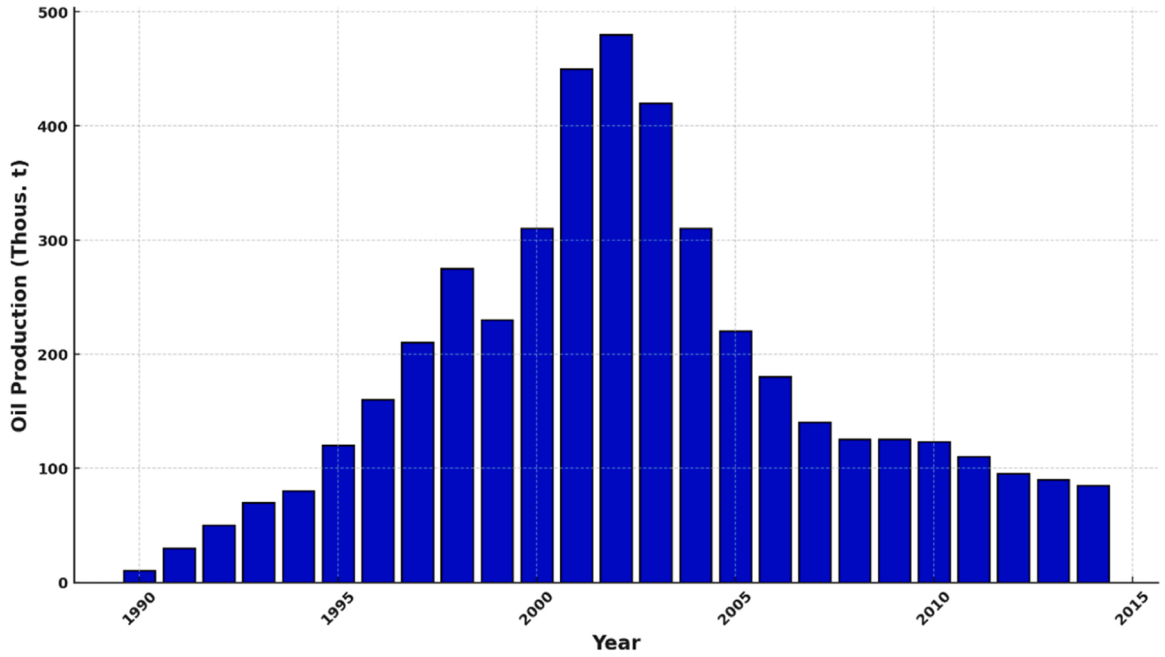

Fig. 3. The bar chart illustrates the annual oil production (in thousand tonnes) from 1990 to 2015. The production shows a steady increase from the early 1990s, peaking around the late 1990s to the early 2000s, followed by a gradual decline.

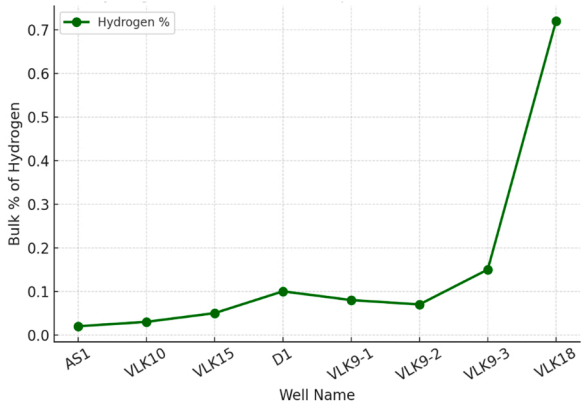

Fig. 4. Distribution of hydrogen in bulk volume of gas collected from different wells.

and structural highs ideal for hydrocarbon accumulation. The tectonic activity has resulted in well-defined traps where hydrocarbons generated in mature Ordovician-Silurian shales have migrated and accumulated. Fields like Pietų Šiūpariai, Degliai, and Pociai are located along these fault systems, with the overlying Silurian shales providing excellent seals. The fields have a long history of hydrocarbon production since the 1990s, most fields are now declining in production and are targeted for secondary recovery (Fig. 3). This oil production data effectively evaluates H_2 production by identifying associated gases and depleted reservoirs as potential H_2 sources. Gas composition trends reveal hydrocarbon-to-H_2 conversion efficiency, while reservoir conditions (temprature 85℃, pressure upto 150 bar, and depth of 2 Km) support microbial H_2 production.

2.2. In-situ gas analysis

Analysis of historical data from the studies conducted while investigating the CO_2 injection in these hydrocarbon fields revealed possibilities of H_2 production. Hence, the gas samples analyzed during the CO_2 injection trial conducted in the fields (Malik et al., 2024) showed the presence of H_2 in the range of 2–20 %. This led to the belief that there is potential for H_2 production from these fields. But to validate this first, gas samples were collected from several different wells using Tedlar bags of 2-liter capacity for gas chromatographic analysis (of O_2, N_2, CO_2, CO, and hydrocarbons (C_1–C_{10}) using an Agilent 7890 A two-channel gas chromatograph (Serial No. CN10181125) with ChemStation software ver. B.04.02. Gas chromatographic analysis showed the presence of H_2 in almost all the samples collected to varying degrees (Fig. 4).

Subsequently, an isotopic analysis was carried out to find the origins of the H_2 gas in the gas chromatographic analysis. The isotopic analysis of gases indicated that the H_2 gas is biogenic. These findings indicated the possible microbial origin of the naturally occurring H_2 in the field. Next, an experimental program was developed to reproduce the process, leading to natural H_2 production in the depleted hydrocarbon fields.

2.3. Extraction of reservoir brine samples

Brine samples (set of 2 for each) were collected from eight different hydrocarbon prodiction wells in sterile 1-liter vials (Fig. 5). Nitrogen was flushed into the vials during the brine sample collection to inhibit the presence of oxygen. The vials were filled with brine and sealed tightly. Thereafter, the samples were transported to the laboratory in disposable heat packs to provide a constant setting. In-situ analysis was performed to collect the pH, salinity, and temperature data onsite for contextualizing microbial activity. The collected samples were utilized for metabarcoding and microbial analysis at the earliest to get accurate findings.

2.4. Metabarcoding analysis

Metabarcoding was performed to analyze the microbial diversity in the collected samples (U21, AG2, D8, K1, K2, P3, P6, SIUP1) to characterize microbial community and evaluate their potential roles in H_2 production (Vavourakis et al., 2016; Elbehery et al., 2023). Primarily, the collected brine samples were filtered through sterile membranes to concentrate cells. DNA was extracted using Xploregen kit, ensuring effective cell lysis and purification, followed by high-quality PCR

Fig. 5. Collected samples from various reservoirs namely, AG-2, SIUP-1, P3, D8, P6, U21, K2, and K1.

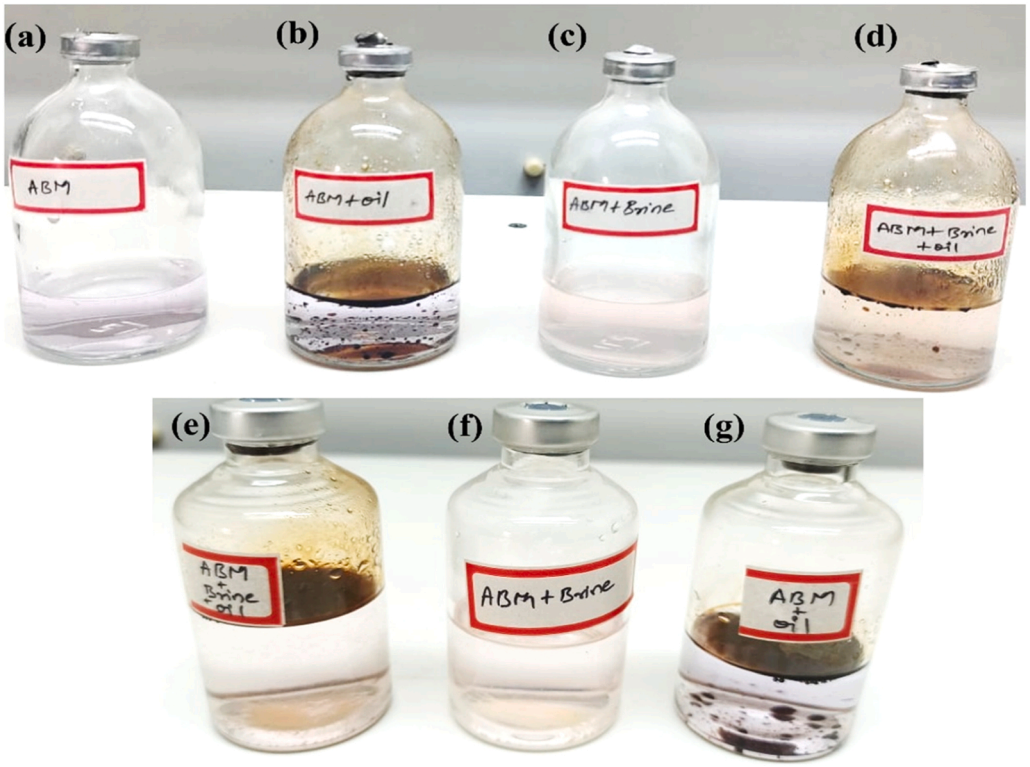

Fig. 6. Photographs of bottles inoculated with: (a) ABM, (b) ABM + oil (2 % v/v), (c) ABM + brine (10 % v/v), and (d) ABM + brine (10 % v/v) + oil (2 % v/v), incubated at 60°C for 84 h; (e) ABM + brine (10 % v/v) + oil (2 % v/v), (f) ABM + brine (10 % v/v), and (g) ABM + oil (2 % v/v), incubated at 60°C for 200 h.

amplification of the V3–V4 region using optimized primers (Eder et al., 2001). The extracted DNA was used in library preparation and sequencing procedures, where it was fragmented and sequencing adapters were added. Sequencing was performed on the Illumina MiSeq platform (2 ×300 bp), and bioinformatics processing included demultiplexing, quality control (FastQC, MultiQC), operational taxonomic unit (OTU) clustering, taxonomic classification via the NCBI 16S rRNA gene profiling database, and comprehensive diversity analysis. Furthermore, data processing and quality control were carried out to exclude low-quality reads, adapters, and host DNA contamination. The

Table 1
Brine sample characteristics.

Sample Name	pH	EC (mS/cm)	TDS (g/L)
D8	4.8	0.624	276.3
P3	4.8	0.2248	112.4
P6	5.5	1.478	2.443
U21	5.1	0.2019	99.24

cleaned data was then examined for taxonomic and functional annotations. Results were visualized through MicrobiomeAnalyst.ca, using heatmap.

2.5. Microbial culture

In the present study, brine samples and oil were mixed with ABM solution at 60°C or 80°C with the optimum pH range of 6.0–7.0 for microbial culture growth. The ABM recipe was selected based on its prevalence in the literature (Dehority and Grubb, 1976; Tanner, 2007; Rouf et al., 2017). The ABM was prepared using the following concentrations per liter of distilled water: 1.0 g NH_4Cl, 0.1 g $CaCl_2{\cdot}6H_2O$, 0.5 g KH_2PO_4, 0.33 g KCl, 0.33 g $MgCl_2{\cdot}6H_2O$, and 0.001 g resazurin. Additionally, 1.0 mL of a trace element solution was added, which contained 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4$, 0.5 g $MnSO_4$, 1.0 g NaCl, 0.1 g $FeSO_4$, 0.1 g $CaCl_2$, 0.1 g $CoCl_2$, 0.1 g $ZnSO_4$, 0.01 g $CuSO_4$, 0.01 g $Al(SO_4)_2$, 0.01 g H_3BO_3, and 0.01 g Na_2MoO_4 per liter of distilled water. To enhance bacterial growth and enzymatic activity, 1.0 mL of a vitamin solution with 2.0 mg biotin, 2.0 mg folic acid, 2.0 mg pyridoxine hydrochloride, 10.0 mg riboflavin, 5.0 mg thiamine, 5.0 mg nicotinic acid, 5.0 mg pantothenic acid, 0.1 mg vitamin B_{12}, 5.0 mg p-aminobenzoic acid, and 5.0 mg thioctic acid was added per liter of distilled water. The medium's pH was adjusted to 7 with 0.1 M HCl, and oil (2 % v/v) served as both a carbon and energy source. The N_2-purged ABM, oil, and brine combination was dispersed into sterilized 100 mL crimp-top bottles with a 30 mL working volume. The headspace was flushed with pure N_2 gas and sealed with a rubber septum and aluminum crimp caps under aseptic anaerobic conditions using an anaerobic chamber (Fig. 6).

All experiments were carried out in triplicates. The first set of bottles (Figs. 6a-6d), incubated for 84 h, includes the following conditions: one bottle containing only ABM, another containing ABM with 2 % (v/v) oil, a third containing ABM with 10 % (v/v) brine, and a fourth containing ABM with both 10 % (v/v) brine and 2 % (v/v) oil. The second set of bottles (Figs. 6e and 6f), incubated for a longer duration of 200 h, consists of bottles with ABM and both 10 % (v/v) brine and 2 % (v/v) oil, ABM with 10 % (v/v) brine only, and ABM with 2 % (v/v) oil only. All the cultures were incubated at 60 or 80 °C under atmospheric

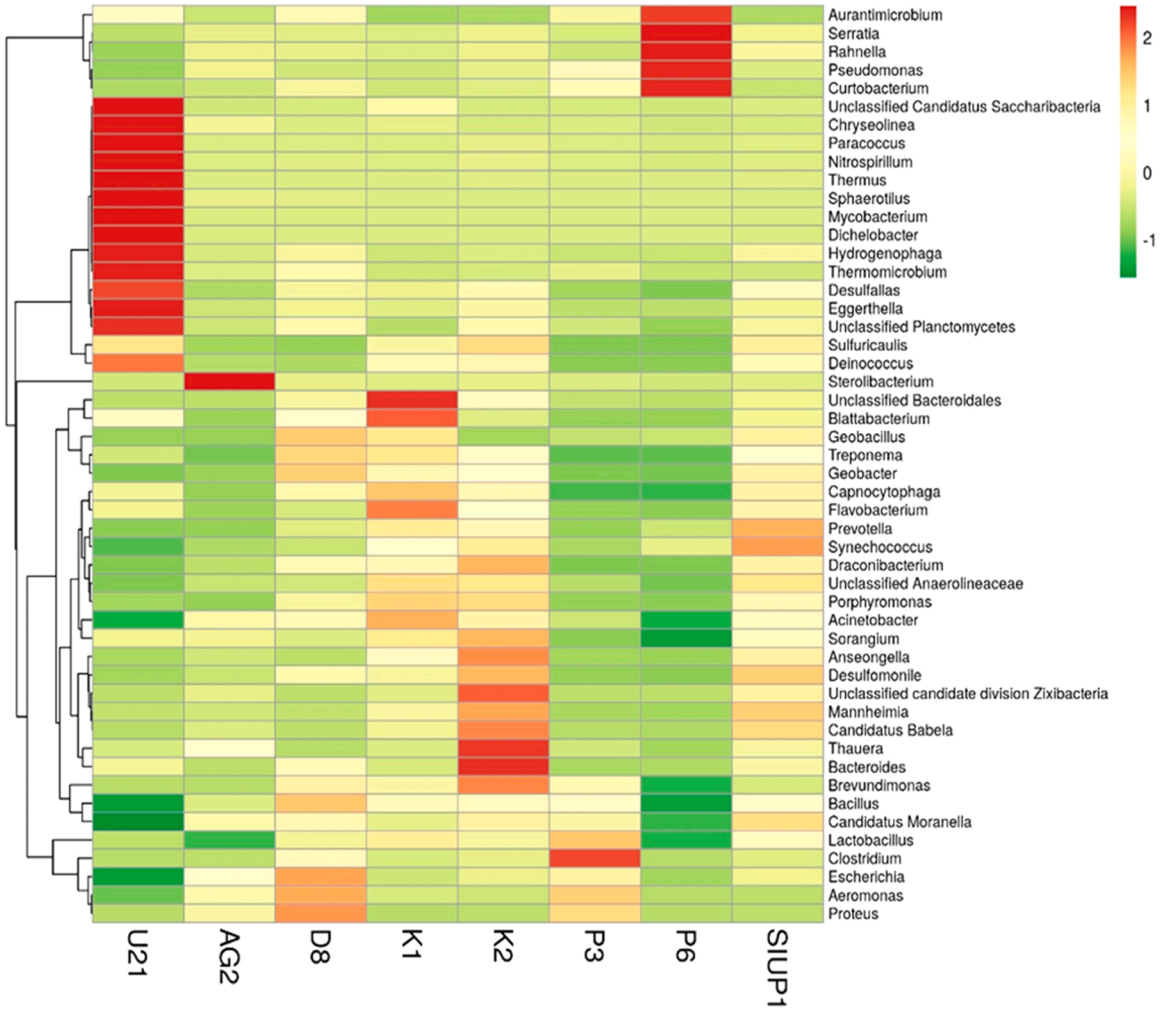

Fig. 7. Hierarchical clustering heatmap of microbial taxa relative abundance across experimental brine samples collected from eight different well sites. The values shown in the heatmap reflect relative abundance of microbial taxa, scaled using unit variance normalization (z scores). Each microbial genus's abundance was normalized across all samples by subtracting the mean and dividing by the standard deviation (i.e., z score transformation). As a result, the units shown are dimensionless, and the color gradient represents how much more or less abundant a genus is in a particular sample compared to its average presence across all samples.

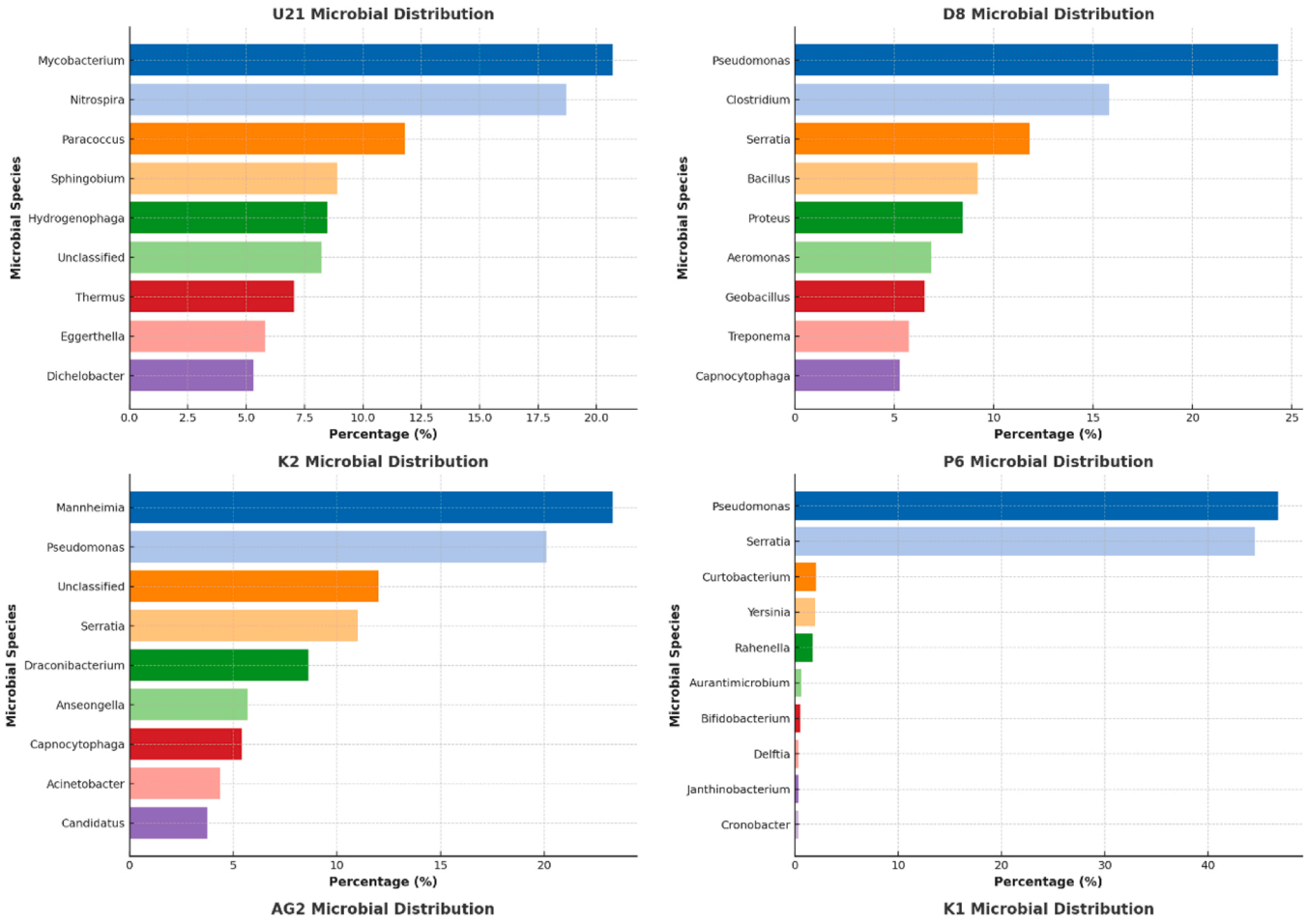

Fig. 8. Figure showing the relative percentage of microbial diversity seen in different brine samples collected from the wells.

pressure, and cells were visualized under an inverted microscope. The growth profiling was done by examining the turbidity by measuring optical density at 600 nm (OD 600 nm) using a UV/VIS spectrophotometer.

2.6. H_2 production analysis

For the H_2 production, 3.3 % (v/v) of the culture was used as inoculum from the microbial culture (after 72 h) with ABM, brine (10 % v/v), and different concentrations of oil (1, 2.5, 5, 10 and 20 % v/v). Using the previously mentioned aseptic anaerobic conditions, the media and inoculum were distributed into 100 mL crimp-top bottles, crimped, and incubated at 60 or 80 °C under atmospheric pressure. The gas samples (2 mL) from the headspace of the bottles were analyzed using Gas Chromatography (GC, Agilent 7890B GC System) on the experiments' 2nd, 4th, 6th, 8th, and 10th days. The GC was equipped with a thermal conductivity detector (TCD); the injector, column, and detector temperatures were 180°C, 40°C, and 200°C, respectively. GC measurements were carried out in triplicates.

3. Results

3.1. Brine characterization

The pH values of the brine samples range from 4.8 to 5.5, indicating acidic conditions like presence of organic acids such as acetic and formic acid. Additionaly, the brine solution contains small immiscible microdroplets of oil, which have a composition like that of crude oil. The crude oil is around 30–40 ° API, the viscosity of the oil is around 1 cp. The Brine samples are of high salinity with salinity ranging from 160K-180K ppm, D8 and P3 brine samples have the lowest pH, suggesting higher acidity, while P6 Brine is the least acidic. Electrical conductivity (EC) varies significantly, with P6 Brine showing the highest value (1.478 mS/cm), indicating a higher ion concentration, whereas U21 Brine has the lowest (0.2019 mS/cm), suggesting lower salinity. Total dissolved solids (TDS) are highest in D8-Brine (276.3 g/L), reflecting high mineral content, while P6 Brine has a remarkably low TDS (2.443 g/L) despite its high EC, suggesting a difference in dissolved ion composition. These variations highlight the diverse geochemical nature of the brines, possibly influenced by differences in geological formations, mineral dissolution, and water-rock interactions.

3.2. Metabarcoding

This provides a robust, reproducible workflow for identifying and quantifying microbial taxa associated with bio H_2 production under surface-accessible brine conditions. It lays a foundational understanding of microbial ecology in subsurface fluids and guides future laboratory-scale enrichment and reactor design experiments targeting hydrogen-producing consortia for bioenergy and reservoir applications. Metabarcoding results for U21, AG2, D8, K1, K2, P3, P6, and SIUP1 reservoir brine samples are shown in Fig. 7. Analysis was also carried out in different samples to analyze the relative % of microbial diversity, see Fig. 8. The analysis of microbial taxa based on their H_2 production efficiency, combined with abundance data from the heatmap, highlights several key candidates. With a peak abundance of ~2.0 microbes in P6

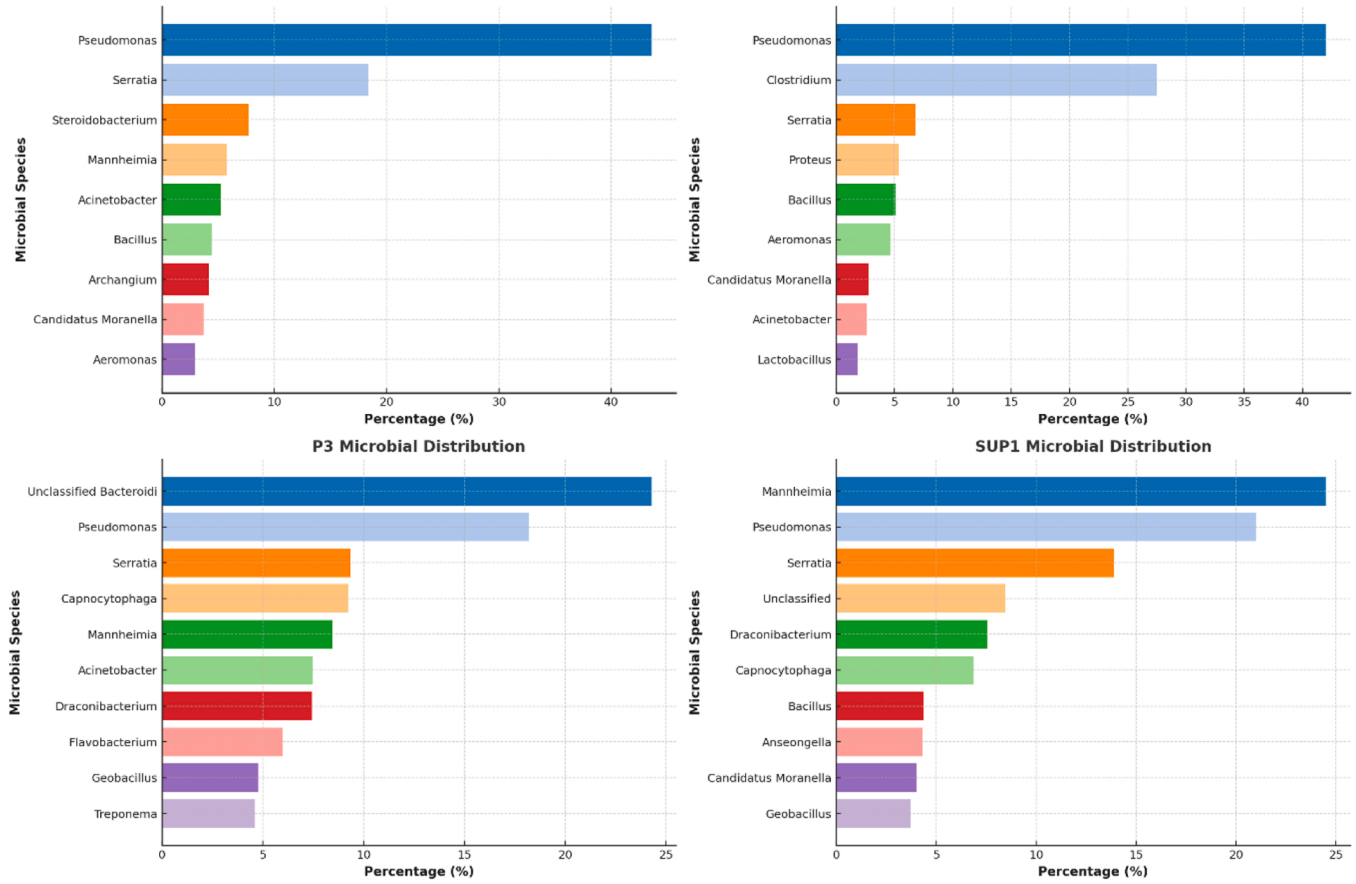

Fig. 8. (*continued*).

such as Aurantimicrobium, Serratia, Rahella, Pseudomonas, and Curtobacterium was most dominant species. While in P3, Clostridium was the most efficient H_2 producer, generating H_2 as a byproduct of anaerobic fermentation. Moreover, Hydrogenophaga, abundant in U21 (~1.5), was another strong spp. due to its utilization of hydrogenases for H_2 production and oxidation. Desulfovibrio (observed as *Desulfomonile*), with moderate abundance in K2, (~0.5), contributes to H_2 generation under sulfate-reducing anaerobic conditions. In contrast, Geobacter, showing relative abundances of ~1.0 in P3, participates in H_2 production through electron transfer mechanisms in anaerobic environments. Thermophilic species like Thermomicrobium, with limited abundance (~-0.3 to-0.6 in K1 and K2), display very less potential for H_2 production in high-temperature conditions, whereas Rahnella, with an abundance of ~1.5 in U21, exhibits H_2 production in nitrate-reducing environments, albeit with lower efficiency. Overall, *Clostridium* and *Hydrogenophaga* was identified as the most efficient H_2 producers, with additional contributions from *Desulfovibrio*, *Geobacter*, *Thermomicrobium*, and *Rahnella*.

Overall, these findings provide quantitative insights into the potential of these taxa for microbial H_2 production and microbial energy applications. This metabarcoding analysis identified the dominant microbial classes involved in H_2 production across different reservoir brine samples, thereby providing valuable insights for laboratory experiments.

3.3. Bacterial culture growth

The growth of the H_2-producing culture in ABM + brine and ABM + brine + oil conditions indicates a significant increase in turbidity after 76 hrs, suggesting active bacterial growth and accumulation of biomass. While incubation up to 200 h showed perceptible turbidity at the bottom of the bottle under ABM + brine + oil and ABM + brine. Bacterial growth phases were determined by measuring the OD at 600 nm for ABM + brine and ABM + brine + oil samples up to 200 h 60°C (Fig. 9). The bacterial cultivation phase showed that ABM + brine (10 % v/v) (Figs. 9a and 9b) and ABM + brine (10 % v/v) + oil (2 % v/v) (Fig. 9c and d) cultures developed noticeable turbidity after 76 h, with the log phase lasting up to 72 h for ABM + brine + oil and 100 h for ABM + brine. In contrast, ABM + oil (2 % v/v) exhibited no perceptible turbidity even after 10 days. For the ABM + brine (10 % v/v) + oil (2 % v/v) culture, 3.3 % (v/v) of the cultivated culture was used as inoculum for the hydrogen production phase after 72 h.

3.4. H_2 production quantification

For H_2 production analysis, all the culture bottles were incubated at 70°C for 10 days, and 2 mL of gas samples were analyzed at regular intervals of 48 h. Fig. 10 compares H_2 and CO_2 production for different concentrations of brine and oil in ABM media incubated at 60°C. The analysis of H_2 and CO_2 production over time across different ABM+-Brine+Oil concentrations (1 %, 2.5 %, 5 %, 10 %, and 20 %) reveals that H_2 production efficiency is concentration-dependent. At lower concentrations (1 %-2.5 %), H_2 levels fluctuate with CO_2 remaining dominant, while higher concentrations (10 %-20 %) show a gradual improvement in H_2 production over time, possibly due to microbial adaptation or equilibrium shifts. Peak H_2 production occurs between 144 and 240 h at higher concentrations, suggesting a delay in optimal conditions for hydrogen generation. Meanwhile, CO_2 production remains relatively stable across all conditions, indicating less sensitivity to concentration changes. This suggests for maximum H_2 production, higher concentrations (10 %-20 %) are preferable, whereas lower concentrations (1 %-5 %) may help minimize CO_2 emissions.

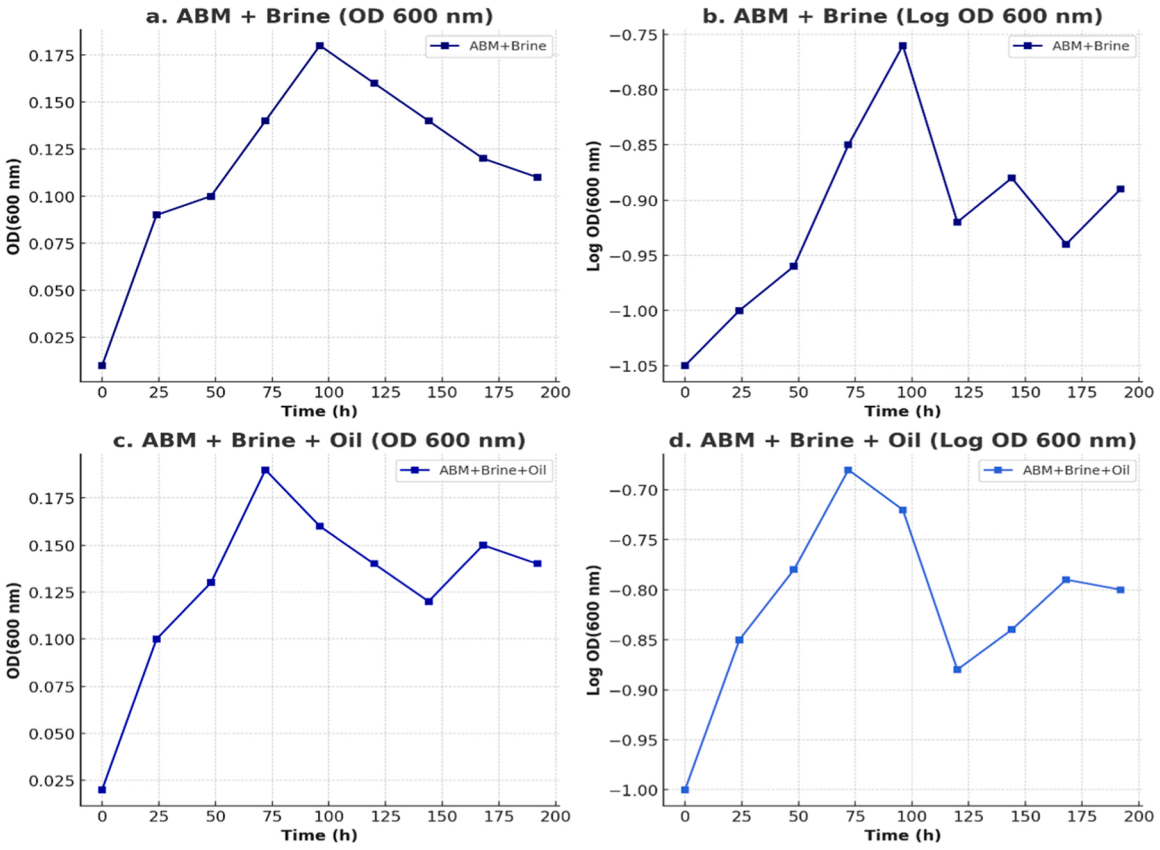

Fig. 9. The plots of OD values at 600 nm for (a) ABM + Brine (10 % v/v) and (b) AMB + Brine (10 % v/v)+ oil (2 % v/v) at 60°C.

H_2 production under different ABM+brine+oil mixtures was observed, presented in Fig. 11. ABM+brine (10 % v/v) + oil (1 % v/v) showed higher H_2 production of 6.5–8 μmol L^{-1}/day at 60°C than other oil and brine mixtures with slight fluctuations. The 2.5 % mixture shows an initial increase, peaking at 96 hrs, followed by a continuous decline. Both the 5 % and 10 % mixes reach a peak at 96 h, then drop with a minor recovery at later times. The 20 % mixture has a distinct pattern, with an initial decrease followed by a strong increase after 192 h, indicating possible delayed microbial activity or response modifications. Higher oil concentrations (>10 %) may first restrict H_2 generation but later promote it. Further investigation is needed to understand microbial and chemical interactions.

4. Discussion

This study provides valuable insights into microbial-driven H_2 production in depleted hydrocarbon sites. Combining brine sample analysis, metabarcoding, and microbial culture studies reveals the crucial function of anaerobic microbial populations in H_2 production. *Clostridium* was found as the predominant H_2 producer, indicating its adaptability and efficiency. The presence of *Desulfovibrio, Geobacter, Thermomicrobium*, and *Rahnella* suggests a complex microbial community that contributes to H_2 production through various metabolic pathways.

The results show that together with Hydrogen CO_2 is also produced, which remains a challenge and needs further optimization of the ABM recipe and the microbial hydrogen production process to either reduce or all together cease the CO_2 production during Hydrogen production. Recent publication by Vilcáez and Chowdhury (2025), have shown a method to produce biogenic hydrogen from oil field with CO_2 being used to produce Hydrogen and CH_4.

These results align with studies by Alotaibi et al., 2020; Boretti and Banik, 2021; Boshagh and Rostami, 2020; Chen et al., 2023; De Vrije et al., 2010; Dehghanimadvar et al., 2020 and Veshareh et al., (2022) emphasizing the significance of *Clostridium* and *Thermotoga* strains in microbial H_2 production. Factors, including pH, temperature, and substrate composition, significantly influenced H_2 production efficiency. Optimal yields were obtained under slightly acidic (pH 4.8–5.5) and high-salinity conditions, which is similar to the findings of Liu and Shen (2004). The highest H_2 yield was observed in cultures with ABM + brine + oil (1 % v/v), demonstrating the importance of oil as a carbon source. The ABM effectively promoted microbial growth and H_2 production, with oil addition enhancing bacterial growth phases, particularly for *Clostridium* and *Hydrogenophaga*. This effect is confirmed by the study of Sugai et al., (2012) who reported hydrocarbons as effective substrates for H_2-producing bacteria. Microbial-driven H_2 production has advantages over traditional technologies like SMR and pyrolysis, including lower energy requirements and lower carbon emissions. However, microbial production rates remain lower than thermochemical processes, emphasizing the importance of further optimizing microbial consortia and growth conditions. Unknown gas byproducts from incomplete metabolic pathways, as well as heterogeneity in H_2 outputs across brine samples, highlight the need for site-specific microbial characterization.

The study identified optimal conditions for microbial H_2 generation, including slightly acidic pH levels (4.8–5.5) and high temperatures (60°C or 80°C), which supported microbial activity and growth. Substrate composition played a crucial role, with 10 % (v/v) brine and 1 % (v/v) oil yielding the highest H_2 production rates (6.5–8 μmol L^{-1}/day at 60°C). While higher oil concentrations (>10 %) initially restricted H_2 generation, they later promoted it, suggesting delayed microbial adaptation. Strict anaerobic conditions maintained using nitrogen-purged media were essential for the growth of obligate anaerobes like Clostridium. Additionally, sufficient incubation time (144–240 h) was necessary to achieve peak H_2 production, highlighting the importance of

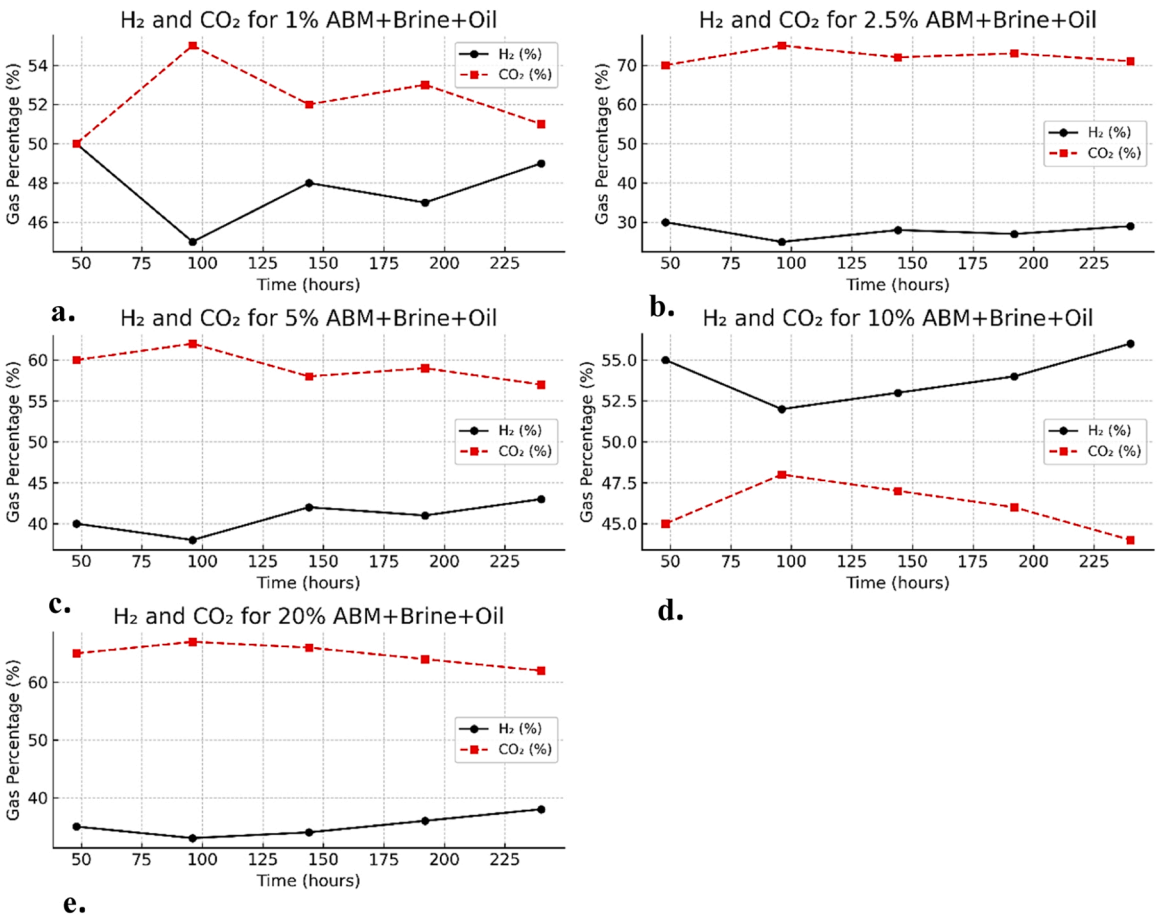

Fig. 10. H_2 and CO_2 percentage over time for different ABM+Brine+Oil media mixtures.

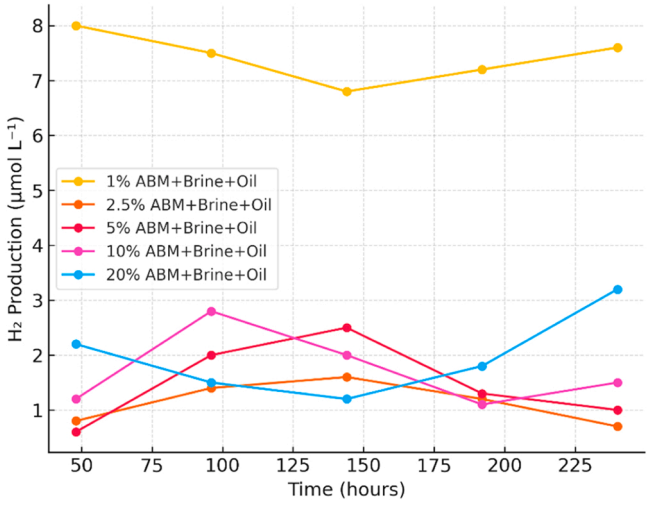

Fig. 11. H_2 production at different time intervals with distinguished ABM-Brine-oil mixtures.

microbial adaptation and activity over time. Together, these conditions optimize microbial-driven H_2 production in depleted hydrocarbon reservoirs.

Future research should focus on genetic engineering to improve microbial H_2 production efficiency, and in-situ field testing to validate laboratory findings under reservoir conditions.

5. Conclusion

This study showed that sustainable H_2 production from crude oil could represent the intersection of conventional energy systems and future-oriented clean energy solutions. This approach can provide a transitional bridge to a low-carbon economy by synthesizing existing research and exploring novel pathways. However, achieving sustainability requires technological innovations, efficient carbon management, and alignment with global decarbonization goals. This study provides a comprehensive understanding of microbial contributions to H_2 production from depleted hydrocarbon fields. The findings emphasize the potential of microbial-driven methods as sustainable and energy-efficient alternatives for hydrogen generation. Additionally, they highlight the feasibility of repurposing depleted oil reservoirs into sustainable energy sources. Overall, the methodology adopted for this study can be used to estimate the H_2 production potential in other potential locations. Notably, brine samples for pH (acidic conditions), high salinity, and TDS revealed favourable conditions for microbial H_2 production. Similar preliminary field parametric characterisation may be useful to identify favourable conditions for microbial activity in other locations. Like this study, metabarcoding of microbial communities in reservoir brine samples can be carried out at other locations as well to identify the major H_2-producing classes. In addition, the study showed that oil serves as an effective carbon source, which enhances microbial growth at high temperatures (60–80°C), particularly for thermophilic microorganisms. The laboratory experiments described in this study can be replicated for other locations as well, such as ABM recipe formula and varying oil concentration to optimize microbial growth for H_2 yield. Similar to this study, monitoring microbial activity and equilibrium shifts in other reservoirs may provide information about the long-term

H_2 production potential. Variations in microbial diversity and H_2 production in saltwater samples suggest the need for site-specific microbial characterization and optimization of growth conditions, which can be applied in other studies. Future work should focus on optimizing microbial consortia, improving production efficiency through genetic engineering, and validating laboratory findings in field-scale trials. Together, these advancements will further position microbial H_2 production as a key contributor to clean energy transitions.

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CRediT authorship contribution statement

Apoorv Verma: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mayur Pal:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Vishwanath R. S:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation. **Mahaveer Kurkuri:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation. **K. Upendranath:** Writing – review & editing, Writing – original draft, Validation, Resources, Investigation, Formal analysis.

Declaration of Competing Interest

Authors declare no conflict of interest.

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Data availability

Data will be made available on request.

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